Pathogen Detection and Remediation for Safe Eating

Biosensor studies of collagen and laminin binding with immobilized *Escherichia coli* O157:H7 and inhibition with naturally occurring food additives

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ABSTRACT

Escherichia coli O157:H7 outbreaks were mostly due to consumption of undercooked contaminated beef which resulted in severe illness and several fatalities. Recalls of contaminated meat are costly for the meat industry. Our research attempts to understand the mechanisms of bacterial adhesion on animal carcass in order to eliminate or reduce pathogens in foods. We have reported the interactions of immobilized *E. coli* O157:H7 cells with extracellular matrix (ECM) components using a surface plasmon resonance biosensor (BIAcore). These studies showed that immobilized bacterial cells allowed the study of real-time binding interactions of bacterial surface with the ECM compounds, collagen I, laminin and fibronectin. Collagen I and laminin bound to the *E. coli* sensor surface with dissociation and association rates ranging from 10^6 to 10^9. Binding of collagen I and laminin mixture resulted in synergistic binding signals. An inhibition model was derived using collagen-laminin as the ligand which binds with *E. coli* sensor. A select group of naturally occurring food additives was evaluated by determining their effectivity in inhibiting the collagen-laminin binding to the bacterial sensor. Bound collagen-laminin was detached from the *E. coli* sensor surface with the aid of an organic acid. The biosensor results were verified with cell aggregation assays which were observed with optical and electron microscopes. These biosensor studies provided understanding of bacterial adhesion to connective tissue macromolecules. It also provided a model system for the rapid assessment of potential inhibitors that can be used in carcass treatment to inhibit or reduce bacterial contamination.

Keywords: *E. coli* O157:H7, SPR biosensor, collagen, laminin, adhesion, inhibition

1. INTRODUCTION

Escherichia coli O157:H7 is an enterohemorraghic pathogen that caused many outbreaks of foodborne illness linked to the consumption of undercooked contaminated ground beef[1]. In addition, several million pounds of ground beef had been recalled recently due to contamination by this organism. The cattle is a reservoir of *E. coli* O157:H7 and the outer surfaces of the beef carcass can be contaminated with *E. coli* during and after slaughter. The surface that lies between the skin and skeletal muscle is composed of the connective tissue and can be the site for bacterial adhesion. Connective tissues in meat such as endomysium, perimysium and epimysium are also components of lean tissues which surround the individual muscle fiber, muscle fiber bundle and muscle sheath, respectively. The mammalian skeletal tissues are composed of actin and myosin which form the muscle fibers. Bundles of muscle fibers are then formed and are subsequently organized into muscle sheaths. Scanning electronmicroscopy of lean meat tissues showed such networks of connective tissues where *E. coli* O157:H7 were found attached[2]. Similar observations were found in our unpublished results. Likewise, the connective tissues of chicken muscle fascia also showed *Salmonella* attachments[3]. Bacterial attachments result from physical entrapment, chemical and biochemical interactions. Extracellular matrix (ECM) proteins such as collagen I, laminin, fibronectin and the glycosaminoglycans (GAGs) are major components of the connective tissues. The interaction between *E. coli* O157:H7 and the connective tissues of animal foods has not been fully elucidated. Understanding the biochemical or biophysical interactions of the bacterial surface molecules with the macromolecules of the connective tissues can lead to development of new intervention approaches to reduce pathogen contamination of beef products. Interactions of the extracellular connective tissue matrix with various *Escherichia coli* strains such enterotoxigenic *E. coli* (ETEC)[4], human enteropathogenic *E. coli* (EPEC) and EPEC strains from rabbits and piglets[5-7] had been reported. Curli-producing strains of

E. coli was reported to bind to fibronectin[8] and another curli-producing *E. coli* O157:H7 also bound to bovine connective tissues[2]. *E. coli* strains isolated from various human infections were shown to bind fibronectin, collagen I, vitronectin and laminin[9-10]. Westerlund and Korhonen[11] reviewed the mechanisms of the binding interactions of the bacterial ECM-binding proteins at the molecular level. These reports suggest that bacterial attachment mechanisms can be elucidated by using the extracellular matrix proteins.

A surface plasmon resonance biosensor (BIAcore) allows direct real-time detection of the binding interactions of macromolecules without chemically altering the ligands for signal generation. The principles of operation, techniques and applications are described in the literature[12-16]. The BIAcore had been utilized to study interactions of ECM macromolecules with surface adhesins of gram positive pathogens, such as the attachment of collagen-binding adhesins of *Staphylococcus aureus* with cartilage and other host tissues[17-18]; the binding interactions of collagens I-VI and fibronectin with recombinant forms of collagen binding surface adhesins and fibronectin adhesins from gram positive bacteria[19]. Immobilized fibronectin was also shown to bind with different fibronectin binding proteins from *S. aureus* and *Staphylococcus epidermidis*[20]. No studies have been reported on the binding interactions of the gram negative bacterial surface with ECMs using the surface plasmon resonance biosensor. In our previous research, the BIAcore was utilized to determine the bacterial surface interactions of the ECM proteins (collagen I, laminin and fibronectin) and hyaluronic acid and chondrotin sulfate[21]. The association and dissociation rates of collagen (k_d=10^{-5} to 10^{-3}; k_a=10^3 to 10^4) and laminin (k_d=10^{-5} to 10^{-3}; k_a=10^3 to 10^4) with immobilized *E. coli* O157:H7 cell surface were also determined. Their affinity constants (K) ranged from 10^6 to 10^9. Fibronectin had no detectable binding with the 5 hr cultured cells but the binding signals increased with the 8 and 24 hr cultured cells. Curli production were also prominent in the latter cultures. Hyaluronic acid and chondrotin sulfate had no detectable interaction with *E. coli* O157:H7 surface and the ECM proteins. The combination of laminin and collagen exhibited a synergistic binding with the bacterial sensor surface while fibronectin reduced the binding of the mixture. Results from this previous investigation provided a model system to study binding interactions of ECM proteins with immobilized *E. coli* cells and indicated that the combination of laminin and collagen also provided optimum binding conditions for inhibition studies.

In this study, we determined the binding interactions of naturally occurring food additives (INI, INL, INK1, INK8 and INL) with the ECM proteins and the immobilized *E. coli* O157:H7 cells. These food additives are classified as "generally regarded as safe" (GRAS) by the Food and Drug Administration. *(The chemical identity of these compounds is being withheld due to a pending patent application.)* Results obtained from this research will be used to study inhibition of bacterial attachment to beef fascia, connective tissues and poultry skin and in the design of methods to control pathogen contamination of meat surfaces.

2. EXPERIMENTAL

2.1. Culture and preparation of cells

E. coli O157:H7 88.1558 (*E. coli* Reference Center, University Park, PA) was maintained on brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI). The bacteria were also grown in BHI broth (Difco) at 37°C with aeration. Five ml of culture grown for 5, 8 and 24 hours were transferred to a 15 ml conical polypropylene tube and centrifuged at 2000*g* for 10 min. The supernatant fluid was aspirated and the packed cells were resuspended in 5 µl of 10 mM sodium acetate buffer (pH 4) for the preparation of the *E. coli* O157:H7 sensor. Another 100 µl of bacterial suspension was transferred to microtiter wells and the optical density (OD) was measured at 405 nm. Cell suspensions with 0.9-1.0 OD (approx. 1×10^9 cfu/ml) were utilized for immobilization.

2.2. Biacore analysis

These studies were performed on the BIAcore 1000 equipped with a BIAlogue command software. The guidelines from the manufacturer (Biosensor, Inc. Uppsala, Sweden) were followed for the preparation of the sensor surfaces, binding and interpretation of the sensorgrams using the BIAevaluation 2.1.

2.2.1. Immobilization. The dextran surface of the flow cells on the sensor chip was activated with 10 µl of a mixture of equal volumes of *N*-hydroxysuccinimide (NHS, 115 mg/ml) and *N*-ethyl-*N*'-(dimethylaminopropyl) carbodiimide (EDC, 750 mg/ml), followed by the injection of the bacteria at a flow rate of 1 µl per min. Aliquots (15 µl) of 5, 8 and 24 h bacterial cell suspensions were immobilized on the surface of Flow Cells 1, 2, and 3, respectively. The remaining activated dextran, not covalently bound with the cell surface, was blocked with 10 µl ethanolamine at a flow rate of 1 µl per min.

Hepes-buffered saline (HBS) pH 7.4, containing 10 mM Hepes, 3.4 mM EDTA, 0.15 M NaCl and 0.005% BIAcore P20 (v/v) was utilized as the running buffer.

2.2.2. ECM macromolecular binding and regeneration. Sodium phosphate buffer (0.1 M, pH 7.2) was utilized as dilution buffer and binding (running) buffer. Guanidine•HCl (0.75M) was acidified with HCl to pH 2.5 and utilized for regeneration of the sensor surface. A chemically characterized collagen I[22] (a gift from Matrix Pharmaceutical, Fremont, CA) was used in this study. Laminin was obtained from Sigma Chemical Company (St. Louis, MO). Collagen I (50 µg/ml) and laminin 50 µg/ml) were analyzed as single compounds and as binary mixtures. Aliquots (200 µl) of collagen I, laminin, and collagen-laminin samples were transferred to sample tubes and 10 µl was injected at a flow rate of 2 µl/min. Binding interactions of the collagen-laminin mixture with *E. coli* surface derived from the 5, 8 and 24 hour cultures were observed. The sensor surface bound with analytes was regenerated by using one pulse (2 µl) of "INH" (0.1 mg/ml) followed by 2 pulses (1 µl each) of 0.75M guanidine-HCl, pH 2.5. Phosphate buffer (0.1 M, pH 7.2) was used as dilution and running buffers[21].

2.2.3. Inhibition with food additives. The food additives (INI, INL, INK1, INK8 and INL) were evaluated at 50 and 100 µg/ml final concentration and their direct interactions with the *E. coli* sensor were evaluated by automatically injecting over the sensor surface. The inhibition experiment was performed by transferring 200 µl of a mixture of 50 µg/ml each of collagen and laminin in phosphate buffer to sample tubes. The food additives were suspended in phosphate buffer at 1 mg/ml concentration and 10 or 20 µl volume was added to the collagen-laminin samples resulting in a final concentration of 50 or 100 µg/ml, respectively. The collagen-laminin and food additive mixture was injected over the sensor surface at 10 ml flow rate of 2 µl/min. The binding interactions were evaluated on sensor surfaces immobilized with 8 or 24 h cultured *E. coli*. The regeneration or removal of captured molecules from the sensor surface bound with analytes was modified by using one pulse (2 µl) of "INH" or "INL" (0.1 mg/ml) followed by 2 pulses (1 µl each) of 0.75 M guanidine.HCl, pH 2.5. In two separate experiments, INH or INL was used to mediate detachment of collagen-laminin from *E. coli* sensor surfaces. The percent inhibition was determined by subtracting the Response Units (RUs) of the samples treated with the food additives from the total RU of collagen-laminin binding to the *E. coli* sensor surface. The difference in RU is then derived from the collagen-laminin binding RU and multiplied by 100.

2.3. Cell aggregation assay and electron microscopy

E. coli O157:H7 was grown in BHI broth. Five ml aliquot of a 16 hr culture was transferred to 15 ml conical centrifuge tubes and the cells were centrifuged for 10 min (ca 2000*g*) in an IEC Model CL Centrifuge (International Equipment Co., Needham, MA). The supernatant was aspirated and discarded. The packed cells were reconstituted with 5 ml PBS (0.1 M), pH 7.2. The optical density (OD) was determined at 405 nm and 100 ml cell suspension with approximately 1.0 OD were utilized. The cell aggregation assay reported by Sanderson et al.[3] was modified for this study. Fifty microliter of *E. coli* suspension (ca 10^9 CFU/ml) was transferred to 12 sterile microtiter wells. To two wells, 50 µl PBS was added and 50 µl of collagen (1 mg/ml) was added to six wells; 50 µl of 1 mg/ml of INL or INK inhibitors was added to four wells with and without collagen. The wells were covered and gently mixed for 10 minutes at room temperature and incubated at 4°C for 16 hrs. The optical density (405 nm) were determined before and after incubation. The samples (40 µl) were transferred to glass slides and covered with glass slips for examination under an Olympus BH-2 microscope. For examination in a scanning electron microscope, samples (5 µl) were transferred to round coverslips. The samples were allowed to adhere to the glass surface for 60 s prior to fixation in a buffer containing 2% glutaraldehyde in 0.1 M imidazole-HCl. The sample on glass cover slips were critical-point dried, mounted on aluminum stubs and sputter coated with a thin layer of gold. The surface topography was observed in a secondary electron imaging mode at 5kV accelerating voltage using JEOL USA Model JSM 840A scanning electron microscope. Digital images were collected from 100× to 10,000× magnification.

3. RESULTS AND DISCUSSIONS

A typical sensorgram of the *E. coli* immobilization is shown in Figure 1. The immobilized 24 hr cell culture generated 425 RU (Response Units). As shown in our previous studies the signals generated were typically higher with cells taken from the stationary phase than with those from the lag and exponential phases. However, the ECM binding responses were inversely proportional to the culture period and RUs of the immobilized bacterial surface[21]. In the current study, the immobilized 5, 8 and 24 hr cell cultures generated 164, 291 and 465 surface RUs of sensor surface. The 5 and 8 hr cultures were used undiluted while the 24 hr culture was diluted 1:2 by volume. The binding kinetics of collagen using these bacterial sensors are shown in Table 1. PBS buffer generated binding signals from 11-28 RUs. The association and disassociation rate constants were fitted from the experimental sensorgram data (as shown in Figure 2) using the Type 1 Model

$$A + B \underset{Kd}{\overset{Ka}{\rightleftharpoons}} AB$$

of the BIAevaluation software. The affinity constant K was determined from ka/kd.

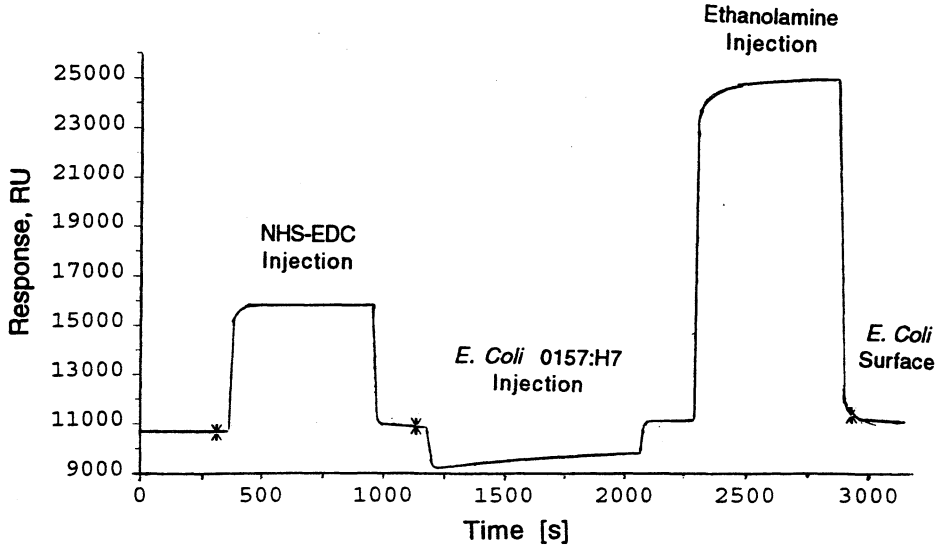

Figure 1. Sensorgram of the immobilization of *E. coli* O157:H7 onto a CM5 sensor chip. The carboxyls of the dextran surface on the sensor chip were activated with a mixture of NHS and EDC followed by the injection of 10 µl bacteria at a flow rate of 1 µl/min. The residual active carboxyls were blocked with ethanolamine. A 20 µl volume of NHS-EDC mixture and ethanolamine were injected at a flow rate of 2 µl/min.

Table 1. Kinetics of collagen binding with *E. coli* O157:H7 surface.

Culture hr	*E. coli* Surface RU	Collagen Bound RU	Dissociation Rate Constant k_d (s^{-1})	Association Rate Constant K_a (M^{-1} S^{-1})	Affinity Constant (K) k_a/k_d (M^{-1})
5	164	1558	1.14×10^{-3}	$.42 \times 10^{4}$	1.24×10^{7}
8	291	1431	1.20×10^{-3}	1.27×10^{4}	1.05×10^{7}
24	465	1404	1.38×10^{-3}	1.38×10^{4}	1.20×10^{7}

We had reported[21] that the mixture of collagen and laminin generated a synergistic binding response compared to the binding of individual collagen or laminin only. Typical sensorgrams of these interactions are shown in Figure 2. In separate experiments using 8 and 24 hr sensor surfaces, the respective dissociation rates of collagen-laminin mixture were 1.25×10^{-3}, 1.75×10^{-3}, 1.36×10^{-3} and 1.40×10^{-3}, 1.49×10^{-3}, 1.51×10^{-3}. Although the binding signals increased, the "off rates" of collagen-laminin mixture were not significantly different from the "off rates" of collagen shown in Table 1. Therefore, the collagen-laminin mixture was used in the inhibition study instead of using collagen alone.

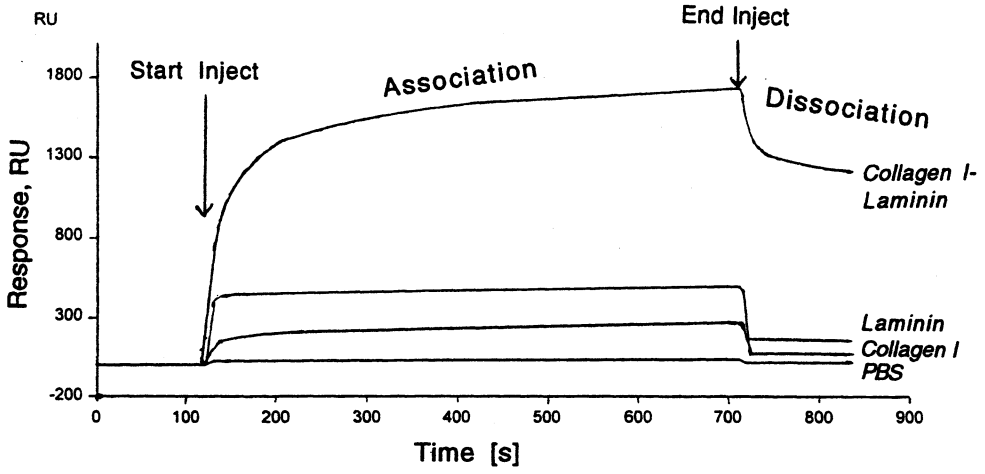

Figure 2. Overlay sensorgrams showing the binding interactions of laminin (LM), collagen I (CI) and collagen-laminin (CI-LM) mixture on the bacterial surface. The approximate molecular weights of laminin and collagen are (M_r = 800,000) and (M_r=290,000), respectively. PBS (phosphate buffer) was used as the running buffer and as a blank sample. The sensor surface was immobilized with *E. coli* which had been cultured for 24 h. RU between the start and end of sample injection indicate the total molecular mass detected by the photodiode array but the captured molecular mass is indicated after sample injection.

INI, INK1, INK8 and INL showed no direct binding with the immobilized bacterial surface as indicated by baseline signals which remained constant and were identical with the phosphate buffer signal. However, when these compounds were mixed with the collagen-laminin mixture prior to injection into the BIAcore, these compounds blocked the binding of the ECMs with the *E. coli* sensor surface. Typical sensorgrams (Figure 3) show these interactions. Further experiments (two trials) demonstrated the inhibition of collagen-laminin binding to the 8 and 24 hr bacteria surface by 50 and 100 µg/ml of inhibitors are shown in Table 2.

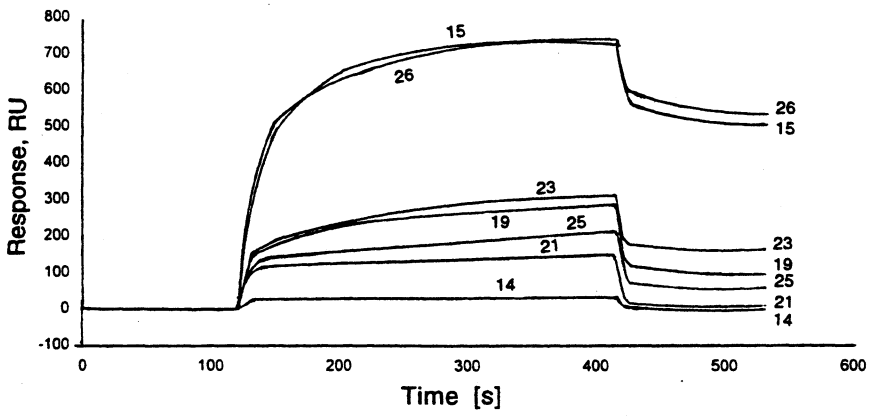

Figure 3. Overlay sensorgrams of the binding responses (RU) on a 24 hr bacterial surface. Binding of collagen and laminin mixture (15, 26) and inhibition by 100 µg/ml INI (19), INL (21), INK1 (23) and INK8 (25) food additives. Phosphate buffer (14) was used as a running buffer and blank sample.

Table 2. Binding RU (Response Units) and % inhibition of collagen-laminin binding to the *E. coli* sensor after treatment with 50 or 100 µg/ml INI, INK1, INK8 and INL compounds.

Sensor Ligands	Inhibitor	A. 50 µg/ml RU **% inhibition**	B. 50 µg/ml RU **% inhibition**	A. 100 µg/ml RU **% inhibition**	B. 100 µg/ml RU **% inhibition**
8 hr					
PBS	none	0	0	0	0
Collagen-laminin	none	732	817	732	817
		0	**0**	**0**	**0**
	INI	322	52	281	6
		56	**94**	**64**	**99**
	INL	16	19	10	3
		98	**98**	**99**	**99.6**
	INK1	432	223	209	61
		37	**73**	**73**	**93**
	INK8	191	92	33	2
		74	**89**	**96**	**99.8**
24 hr					
PBS	none	6	6	6	6
Collagen-laminin	none	514	313	514	313
		0	**0**	**0**	**0**
	INI	**227**	45	100	14
		56	**86**	**80**	**55**
	INL	**27**	27	13	31
		95	**91**	**97**	**90**
	INK1	343	137	167	43
		33	**56**	**68**	**86**
	INK8	189	59	63	21
		63	**79**	**88**	**93**

The results in Table 2 indicate that 50 µg/ml of the food additives blocked 33 to 98% (Mean = 73%) of the collagen-laminin binding to the bacterial sensor surface. Inhibition of collagen-laminin binding ranged from 55 to 99.8% (Mean=86%) when the food additives were used at 100 µg/ml level. Individual food additives (INL, INK8, INI and INI) had mean inhibition of 96, 85, 74 and 65%, respectively. Variability in responses were less in the more reactive INL and INK8 than with INI and INK1. INL had similar inhibitory effect at 50 µg/ml to that of 100 µg/ml whereas the activity of INK8, INI and INK1 increased at the higher concentration of 100 µg/ml. Binding responses were lower with the 24 hr than with the 8 hr sensor. The binding kinetics (association and dissociation rates) of these compounds with collagen are currently being assessed. The mechanisms of collagen binding with the bacterial surface and how these food additives inhibit this binding will also be proposed in our future studies. Other similar compounds will also be assessed for their inhibitory and detachment abilities. These "inhibitors" will also be utilized in inhibition and detachment studies of the food borne pathogens inoculated on beef fascia and poultry skin.

Examination with an optical microscope (40×) of the interactions of ECM, *E. coli* O157:H7 and the food additives, INK and INL indicated the formation of aggregated cells with ECMs (collagen and laminin) and the bacterial cells. The bacteria attached to collagen aggregates and covered the collagen surface. Due to high cell density, there was an excess of unattached *E. coli* cells. Addition of INK and INL to the ECM-bacteria mixture inhibited cell aggregation. The cell distribution in the latter was similar to the control samples (without ECMs). Further examination of these interactions with a scanning electron microscope showed the distribution of untreated *E. coli* cells (Figure 4A) which also showed self aggregation in longer culture periods as in the 8 and 24 hr cultures. This self-aggregation is different from the aggregation resulting from bacterial attachment to the collagen and laminin (Figure 4B). The effects of the INK and INL inhibitors are shown in Figures 4C and 4D. The presence of these compounds inhibited the bacterial attachment to collagen and laminin. These observations with the optical and electron microscopes agree with the biosensor results showing the inhibition of collagen-laminin binding to *E. coli* surface. However, both optical and electron microscopic studies indicated that INK8 had greater inhibitory effect then INL.

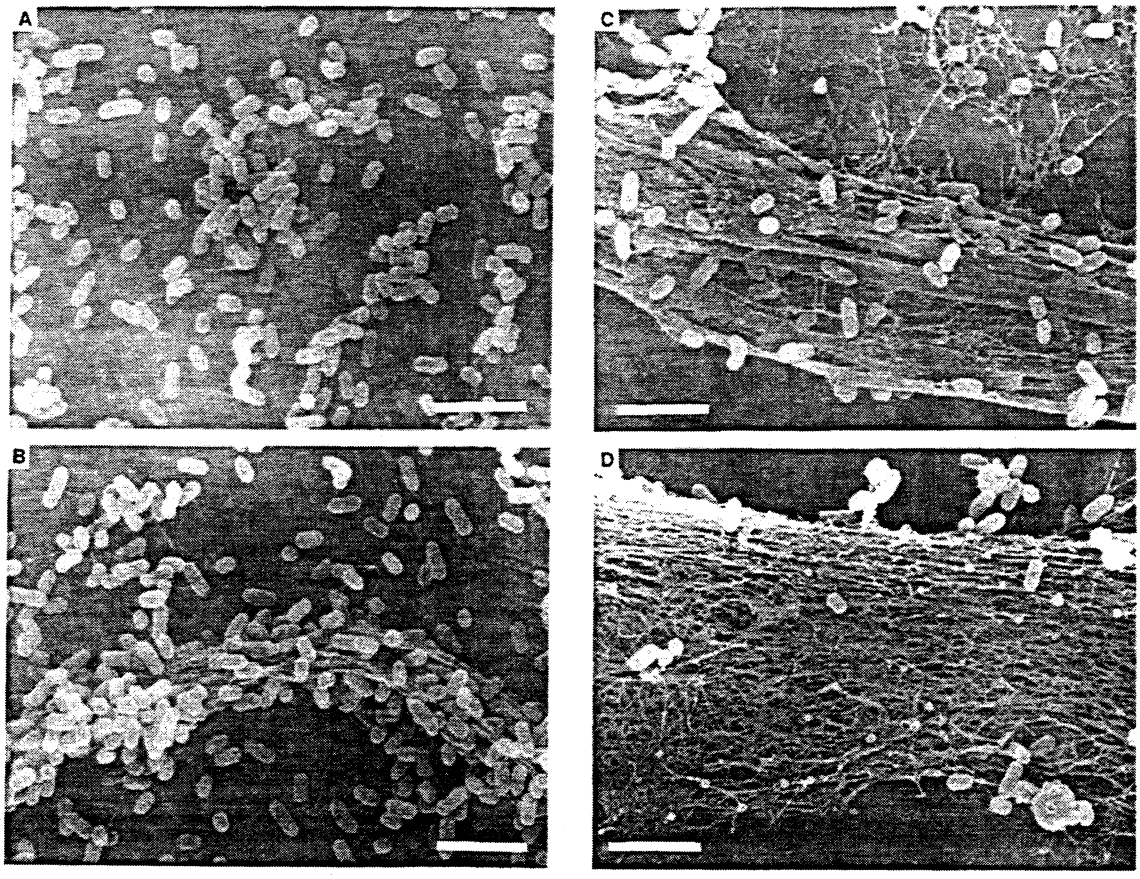

Figure 4. SEM micrographs (scale bar = 2.5 μm) of the interactions of *E. coli* O157:H7 with collagen-laminin mixture and INL and INK inhibitors. A: Self-aggregation of untreated *E. coli* O157:H7. B: Attachment of *E. coli* O157:H7 to collagen-laminin mixture. C: INL blocked *E. coli* attachment to collagen-laminin. D: INK8 blocked *E. coli* attachment to collagen-laminin.

4. CONCLUSIONS

The SPR biosensor provided a model system to analyze the binding of collagen I, and laminin with immobilized *E. coli* O157:H7cells and their interactions with food additives having inhibitory potentials. The collagen I-laminin mixture also provided reproducible and quantitative interactions with the bacterial sensor surface and the "inhibitors". Our biosensor studies indicated that the compound "INL" exhibited the highest inhibitory effect followed by INK8, INI and INK1. This model system led us to assess the effectiveness of the select compounds to block attachment to or detach food pathogens from tissues. Successful completion of this research can generate novel processing treatment to reduce pathogen contamination of animal foods.

5. ACKNOWLEDGMENTS

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6. REFERENCES

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